

# MiR-450 inhibits cell proliferation, migration and invasion and induces apoptosis in gastric cancer via targeting CREB1

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## Primary research

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# Abstract

**Background**—Gastric cancer seriously affects human health and research on gastric cancer is increasing. In recent years, molecular targets are hot research topics. We aimed to explore the effects and mechanisms of miR-450 on the development and progression of gastric cancer.

**Methods**—We used gain-of-function approaches to investigate the gastric cancer cell proliferation, apoptosis, migration and invasion, including, RT-qPCR, CCK-8, colony formation, flow cytometry, western blot, wound healing, transwell chamber, HE, TUNEL, dual luciferase reporter and tumor formation analysis.

**Results**—We found that the expression levels of miR-450 were greatly decreased in gastric cancer cells and overexpression of miR-450 inhibited the gastric cancer cell proliferation, migration and invasion, while induced apoptosis in gastric cancer *in vitro*. Moreover, we demonstrated that ectopic expression of miR-450 inhibited tumor growth *in vivo*. At the molecular level, overexpression of miR-450 significantly increased the expression levels of apoptosis-associated proteins, including Caspase-3, Caspase-9 and BAX, while inhibited the expression level of Bcl-2. Mechanically, luciferase reporter experiment suggested that CREB1 had a negative correlation with miR-450 expression and knockdown of CREB1 alleviated gastric cancer. Furthermore, we also found that miR-450 inhibited the activation of AKT/GSK-3 $\beta$  signaling pathway to inhibit the progression of gastric cancer.

**Conclusions**—miR-450 repressed gastric cancer cell proliferation, migration and invasion and induced apoptosis through targeting CREB1 by modulating AKT/GSK-3 $\beta$  signaling pathway, which may provide a new molecular target for the treatment of gastric cancer.

## Background

Human gastric cancer is one of the common malignancies around the world [1]. Approximately 850,000 cases of gastric cancer are diagnosed and 650,000 deaths occur each year [2]. Different treatment strategies, including surgery, chemotherapy, and radiation, have achieved remarkable advance. However, overall therapeutic activity for advanced disease remains poor [3]. Thus, it is critical to acquire a better understanding of molecular mechanisms and new treatment strategies urgently need to be developed for improving the treatment of gastric cancer patients.

MicroRNAs (miRNAs) are a kind of endogenous small RNA with a length of about 20–24 nucleotides, which have a variety of important regulatory roles in cells. New research showed that miRNAs were involved in the development and regulation of gastric cancer. MiR-4317 inhibits cell proliferation and blocked the conversion of S-G2/M in gastric cancer cells, promising a therapeutic molecular target [4]. MiR-455 suppresses cell proliferation of gastric cancer and migration by modulating EGFR, acting as a potential target for treatment of gastric cancer [5]. MiR-582-5p inhibits cell proliferation and promoted apoptosis by regulating AKT3 [6]. Other studies have also shown the inhibitory effect of miRNAs on the progression and development of gastric cancer, including miR-744, miR-140-5p, miR-181a, miR-182, miR-

802, miR-21, miR-149 [7–13]. On the other hand, miR-450, as a novel miRNA, which function as a tumor suppressor in the regulation of glaucoma [14]. However, its specific role in the gastric cancer and the underline mechanism remain unknown.

Based on its anti-cancer role previously, we hypothesized that miR-450 may act as a tumor-suppressor to regulate the development and progression of gastric cancer. In the present study, we found that the expression levels of miR-450 were significantly decreased in gastric cancer cells. Overexpression of miR-450 inhibited cell proliferation, migration, and invasion and induced cell apoptosis in gastric cancer in gastric cancer *in vitro*. Moreover, overexpression of miR-450 inhibited the growth of tumor *in vivo*. Furthermore, we found that CREB1 served as the molecular target and mediated all the anti-tumor effects of miR-450. Taken together, these results indicates that miR-450 may is a potential molecular target in the treatment of gastric cancer.

## Materials And Methods

### Cell culture

The human gastric cancer cell lines (BGC-823, SGC7901) and human gastric epithelial cell (GES-1) were purchased from the Cell Bank of the Chinese Academy of Science (Shanghai, China). Cells were maintained in Dulbecco's modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS, Gibco, NY, USA) in a humidified air at 5% CO<sub>2</sub> atmosphere at 37°C.

### RNA extraction and real-time quantitative PCR (RT-qPCR)

RNA was isolated by Trizol (Invitrogen, CA, USA) according to the manufacturer's protocols. RNAs were reverse transcribed to cDNA by employing PrimeScript RT Master Mix (Takara, Dalian, China) following the manufacturer's protocol. PCR amplification was conducted with the SYBR Premix Ex Taq™ Kit (Takara, Dalian, China). GAPDH and U6 were used to normalize the expression. The relative expression was calculated using the  $2^{-\Delta\Delta Ct}$  method.

### Oligonucleotides and transfection

The plasmid carrying the CREB1 CDS domain was used to overexpress the CREB1 in gastric cancer cells and a pcDNA (pcDNA3.1) vector was used as a negative control (GenePharma, Guangzhou, China). The cDNA encoding CREB1 CDS domain was amplified by PCR, and then subcloned into the pcDNA3.1 vector (Invitrogen, CA, USA) to obtain the pcDNA-CREB1. The miR-450 mimic and the negative control (NC) were synthesized by GenePharma (Guangzhou, China). The transfection was performed by using Lipofectamine 3000 Reagent (Life Technologies, Carlsbad, CA, USA).

### Cell proliferation assay

Cell Counting Kit-8 (CCK-8) and colony formation assays were performed to examine gastric cancer cell proliferation. In brief, BGC-823 or SGC7901 cells ( $1 \times 10^4$ /well) were seeded in 96-well plates and seeded

for 0 h, 24 h, 48 h and 72 h, respectively. Then BGC-823 or SGC7901 cells were incubated with 10  $\mu$ L CCK-8 solution for 4 h at 37°C. The optical density (OD) was recorded using a microplate at 490 nm (Multiskan MK3, Thermo Scientific, USA). For colony formation assay, BGC-823 and SGC7901 cells ( $2 \times 10^4$  cells/well) were seeded in 24-well plates. After incubation for 12 days, the cells were immobilized with paraformaldehyde for 30 min, and stained with 10% crystal violet for another 30 min. Colonies were counted and photographed with a light microscope (Olympus, Tokyo, Japan).

#### Cell apoptosis assay

Flow cytometry was conducted to investigate the gastric cancer cell apoptosis. Cells ( $1 \times 10^4$  cells/well) were seeded in six-well plates and culture for 48 h. Thereafter, cells were washed using PBS, treated with 5  $\mu$ L of annexin V-FITC and 5  $\mu$ L of PI in the dark for 15 min at room temperature. Then apoptosis cells were detected through a FACScalibur Flow Cytometry (BD Biosciences, CA, USA).

#### Cell migration and invasion assays

Wound healing and transwell chamber (5- $\mu$ m pore size, Costar, Cambridge, MA, USA) assays were conducted to examine gastric cancer cell migration and invasion abilities. For wound healing assay, cells were seeded into six-well plates. The supernatant fluid was removed when BGC-823 or SGC7901 cells were highly confluent (> 90%). Scratches were made using a sterile pipette tip, with the scratch width remaining the same. After continuous culture for 48 h, the width of the scratch was photographed and recorded under a microscope ( $\times 100$ ). For invasion assay, BGC-823 or SGC7901 cells were estimated by transwell assays. In short, transfected BGC-823 or SGC7901 cells were added to the upper chamber loaded with matrigel (Corning, Cambridge, MA) and the bottom of the chamber were supplemented with complete medium containing 1% FBS. 48 h later, cells on the surface of membranes were wiped out. Invaded cells were fixed in 10% formaldehyde, dyed with 0.1% crystal violet and then counted with a light microscope (Olympus, Tokyo, Japan).

#### Hematoxylin-eosin staining (HE) assay

Tumor slices were stained with hematoxylin for 5 min, then rinsed for 1 min, and returned to blue by 1% ammonia (30 s). Afterwards, slices were flushed with running water (1 min). Furthermore, slices were stained by 0.5% H&E (for 1 min), rinsed (for 30 s), made into transparent, and finally mounted with neutral gum.

#### Immunohistochemical (IHC) assay

Tumor sample slides were deparaffinized (xylene), rehydrated (ethanol), and incubated with  $H_2O_2$  at 37 °C for 10 min. Tumor sections were incubated with antibody against Ki67 (ab15580; 1: 1000; Abcam, USA) at 4 °C overnight., followed by incubation with the biotin-conjugated goat anti-rabbit immunoglobulin G secondary antibody (ab6721; 1: 1000; Abcam, USA) at 37 °C for another 30 min. DAB was used as chromogenic agent. Images were obtained using a microscope ( $\times 200$ ).

## TUNEL assays

The slices were washed by PBS and then immobilized for 30 min with 4% paraformaldehyde. After washed with PBS once, the slices were added with 0.1% Triton X-100 for 2 min, and then washed with PBS once. Afterwards, 3% H<sub>2</sub>O<sub>2</sub> was used for incubation (5 min). Then the slides was rinsed, and maintained with 50 μL TUNEL at 37 °C overnight. After rinsed by PBS again, the TUNEL reaction was visualized by chromogenic staining with DAB (Sigma-Aldrich). Light microscope was applied to observe the slides and the TUNEL-positive BGC-823 or SGC7901 cells were calculated in Image J software.

## Dual luciferase reporter assay

A putative 3'-untranslated regions (3'-UTR) of CREB1 was mutated using mutagenesis kit (Promega, USA). Wild type and mutant sequences were amplified and inserted into the vector to construct luciferase reporter plasmids according to the manufacturer's instructions (Promega, USA). The luciferase activities were detected with the dual luciferase reporter kit (Promega, USA). Luciferase activity was measured by dual-luciferase reporter assay system (Promega) and presented as firefly luciferase intensity normalized to Renilla luciferase activity.

## Western blotting analysis

Total proteins in tissues or cells were dissolved with RIPA lysate buffer (Beyotime Inc, Shanghai, China). The protein was quantified using BCA protein assay Kit (Thermo Scientific, CA, USA). The protein samples were separated by 12% polyacrylamide gel, which were transferred to the PVDF membrane, sealed with 5% skim milk powder. The membrane was incubated with the primary antibody at 4°C overnight and then incubated with HRP coupled secondary antibody (Santa Cruz Inc, CA, USA) at room temperature for 1 h. The protein signal was detected by ECL detection reagents (Thermo Scientific, CA, USA) and GAPDH worked as the internal reference.

## Xenograft tumors in nude mice

Female nude mice (6-week-old, 18–22 g) were provided by Nanjing Medical University and housed under germ free conditions. Animal care and use were carried out according to the ethical guidelines by the First Affiliated Hospital of USTC Animal Care and Use Committee. Nude mice were maintained in a 12 h light/12 dark cycle in a temperature- and humidity-controlled environment. To detect the effect of miR-450 on tumor growth *in vivo*, BGC-823 cells ( $1 \times 10^6$  cells) were injected subcutaneously into the right axilla of the nude mice. Following a 30-day period, nude mice were sacrificed, and neoplasms were isolated for further analyses. Note that the tumor volumes were recorded every week and calculated with the formula: Volume =  $0.5 \times \text{length} \times \text{wide}^2$ .

## Statistical analyses

All data are presented as the mean  $\pm$  SD. Each bar expressed the mean  $\pm$  SD of three independent experiments. Statistical significance between two or multiple groups was analyzed by t-test or one-way

ANOVA using SPSS 17.0 (SPSS Inc, Chicago, IL, USA). Experiments were repeated three times independently. Statistical significance was assumed when  $P < 0.05$ .

## Results

### Effect of miR-450 on gastric cancer cell proliferation and apoptosis

RT-qPCR was employed to screen the expression level of miR-450 in gastric cancer cells. As shown in Fig. 1A, the expression levels of miR-450 were significantly decreased in gastric cancer cells (BGC-823 and SGC7901), compared with that in human gastric epithelial cell (GES-1). To further evaluate the roles of miR-450 in the regulation of gastric cancer, gain-of-function assays were implemented in BGC-823 and SGC7901 gastric cancer cells, respectively. The efficiency of transfection was validated by RT-qPCR (Fig. 1B). More importantly, the results of CCK-8 and colony formation assays indicated that the cell viability and number of colonies were significantly decreased in BGC-823 and SGC7901 cells which transfected with miR-450 mimic, compared with that in NC mimic group (Fig. 1C, 1D). Subsequently, flow cytometry assay were conducted to examine cell apoptosis of gastric cancer. We found that cell apoptosis rate was significantly increased in miR-450 mimic group, compared with NC mimic group (Fig. 1E). Consistent with these findings, western blot analysis revealed that the expression levels of apoptosis-associated proteins, including Caspase-3, Caspase-9 and Bax, were significantly increased, whereas Bcl-2 was decreased in miR-450 mimic-transfected BGC-823 and SGC7901 cells (Fig. 1F). These results indicated that miR-450 overexpression inhibited cell proliferation and induced cell apoptosis of gastric cancer.

### Overexpression of miR-450 inhibits tumor growth *in vivo*

To confirm whether miR-450 inhibit the tumor growth of gastric cancer *in vivo*, we generated BGC-823/miR-450 mimic cells and their negative control, then injected them into nude mice. Representative images of tumor in the nude mice with miR-450 mimic and NC mimic (Figure. 2A). Tumor volume and tumor weight were significantly decreased in miR-450 mimic group compared with that in NC mimic group (Figure. 2B, 2C). Furthermore, HE and IHC assays showed that the cell proliferation gene of Ki67 was significantly inhibited in miR-450 mimic group compared with that in **NC mimic group. Furthermore, the results of TUNEL assay indicated that the cell apoptosis was** significantly promoted in miR-450 mimic group compared with that in NC mimic group (Figure. 2D). Consistent with these findings, western blot assay suggested that the expression levels of apoptosis-associated proteins, including Caspaes-3, Caspase-9 and Bax, were significantly decreased in miR-450 mimic group compared with that in NC mimic group, contrary by Bcl-2 (Figure. 2E). These results indicated that overexpression of miR-450 suppressed tumor growth *in vivo*.

### overexpression of miR-450 inhibits gastric cancer cell migration and invasion

Cell migration and invasion play important role in the pathogenesis of cancer metastasis. The results of wound healing assay showed that the wound closure rate was significantly decreased in miR-450-

overexpressed BGC-823 and SGC7901 gastric cancer cells, respectively, compared with that in NC mimic group (Fig. 3A). Moreover, the results of transwell invasion assay showed that ectopic expression of miR-450 limited the inventory capability of BGC-823 and SGC7901 cells, compared with that in NC mimic group (Fig. 3B). Furthermore, western blot assay suggested that the expression levels of migration- and invasion-associated proteins, miR-450 mimic obviously decreased the levels of MMP-2 and MMP-9 in both BGC-823 and SGC7901 cells, compared with that in NC mimic group (Fig. 3C). These data suggested that overexpression of miR-450 inhibits migration and invasion of BGC-823 and SGC7901 cells.

#### MiR-450 targets and negatively regulates CREB1

The expression levels of CREB1 were significantly increased in BGC-823 and SGC7901 gastric cancer cells (Fig. 4A). To study on the possible targets of miR-450 involved in the occurrence of gastric cancer, firstly, TargetScan was carried out to predict that CREB1 was potential candidate of miR-450. We found that CREB1 might be a candidate target of miR-450 (Fig. 4B). Dual luciferase reporter assay was conducted in HEK-293T cells. The luciferase reporter assay showed that miR-450 mimic repressed the relative luciferase activities containing the WT 3'-UTR of CREB1, but had no obvious effect on Mut 3'-UTR of CREB1 (Fig. 4C). Moreover, RT-qPCR and western blot analysis were adapted to evaluate the expression of CREB1 in BGC-823 and SGC7901 cells transfected with miR-450 mimic or NC mimic, and the data revealed that up-regulation of miR-450 decreased the expression of CREB1 (Fig. 4D, E). These results indicated that miR-450 targets and negatively regulates the CREB1.

Knockdown of CREB1 inhibits gastric cancer cell proliferation, migration, invasion and induces apoptosis,

After identifying CREB1 as a target of miR-450 in gastric cancer, we then focused on whether CREB1 regulate the progression gastric cancer. We evaluated the efficiency of knockdown of CREB1 in BGC-823 and SGC7901 cells by RT-qPCR (Fig. 5A). CCK-8 and colony formation assays showed that the cell viability and the number of colonies were significantly decreased in BGC-823 and SGC7901 cells in sh-CREB1 group compared with that in sh-NC group (Fig. 5B, 5C). Subsequently, flow cytometry was conducted to examine cell apoptosis after knockdown of CREB1 expression in BGC-823 and SGC7901 cells and the results indicated cell apoptosis rate was significantly increased in CREB1-knockdowned BGC-823 and SGC7901 gastric cancer cells, compared with that in NC mimic group (Fig. 5D). Wound healing and transwell chamber assays showed that the gastric cancer cell migration and invasion capability was significantly inhibited in CREB1-knockdowned BGC-823 and SGC7901 gastric cancer cells, compared with that in NC mimic group (Fig. 5E, 5F). These results showed that knockdown of CREB1 suppressed gastric cancer cell proliferation, migration and invasion and induced apoptosis.

#### CREB1 overexpression partially restores the effects of miR-450 on gastric cancer

To dissect the role of CREB1 in mediating the inhibitory effects of miR-450 on gastric cancer, overexpression CREB1 in gastric cancer cells transfected with miR-450 mimic. CCK-8 and colony formation assays indicated that the cell viability and the colony numbers of gastric cancer cells were

significantly decreased in BGC-823 and SGC7901 gastric cancer cells in the miR-450 mimic-treated group, when compared with that in NC mimic + pcDNA 3.1 group, while CREB partially abolished the inhibitory effects of miR-450 mimic on cell proliferation of BGC-823 and SGC7901 gastric cancer cells (Fig. 6A and 6B). Flow cytometry assays indicated that miR-450 mimic-induced promotion of cell apoptosis was prominently abrogated by CREB1 overexpression (Fig. 6C).

At the molecular level, western blot assay indicated that the expression levels of apoptosis-associated proteins, including Caspase-3, Caspase-9 and BAX, were significantly increased in BGC-823 and SGC7901 gastric cancer cells treated with miR-450 mimic, compared with that in NC mimic + pcDNA 3.1 group, whereas the overexpression of CREB similarly abolished the activation effects of miR-450 mimic on the expression of apoptosis-associated proteins, including Caspase-3, Caspase-9 and Bax. Coincidence with these results, the expression levels of apoptosis-associated protein of BCL-2 were controversially changed (Fig. 6D). These results pointed out that CREB1 overexpression partially restored the effects of miR-450 on proliferation and apoptosis of gastric cancer.

MiR-450 regulates the gastric cancer progression through inhibiting AKT/GSK-3 $\beta$  signaling pathway

Given the importance of AKT/GSK-3 $\beta$  in the regulation of gastric cancer, thus we determined the protein expression levels of the AKT/GSK-3 $\beta$  pathway by western blot assay (Fig. 7). We found that the expression levels of the AKT/GSK-3 $\beta$  pathway-associated protein, including GSK-3 $\beta$  and AKT, had no significant differences in all the experiments. However, the protein phosphorylation levels were markedly decreased in response to the overexpression of miR-450, while CREB overexpression correspondingly increased AKT/GSK-3 $\beta$  phosphorylation in miR-450 mimic-treated BGC-823 cells. In addition, to further identify the role of AKT/GSK-3 $\beta$  in mediating the anti-gastric cancer role of miR-450, we use a SC79 as an activator of the AKT/GSK-3 $\beta$  pathway. As shown in Fig. 8A, 8B, 8D and 8E, CCK-8 and colony formation, wound healing and transwell chamber assays showed that the gastric cancer cell proliferation, migration and invasion abilities were significantly decreased in the miR-450 mimic-treated group, while significantly increased in the miR-450 mimic + SC79 group compared with that in miR-450 mimic group. These results were further confirmed by flow cytometry, which showed that the apoptosis rate markedly increased in miR-450 mimic group, while significantly decreased in miR-450 mimic + SC79 group (Fig. 8C). The data suggested that miR-450 represses the gastric cancer progression by targeting CREB1 through inhibiting AKT/GSK-3 $\beta$  signaling pathway.

## Discussion

Our findings found that miR-450 was significantly decreased in gastric cancer. Functionally, overexpression of miR-450 inhibited cell proliferation, migration and invasion in gastric cancer *in vitro*, while facilitated cell apoptosis of gastric cancer *in vitro*. Over-expression of miR-450 suppressed the tumor growth *in vivo*. At the molecular level, overexpression of miR-450 increased the expression levels of apoptosis-associated proteins, including Caspase-3, Caspase-9 and Bax, while inhibited the expression level of Bcl-2. Moreover, overexpression of miR-450 suppressed the migration and invasion-associated

protein expression levels, including, MMP-2 and MMP-9. Mechanically, we found that miR-450 alleviated the development and progression of gastric cancer via targeting CREB1 and regulated the AKT/GSK-3 $\beta$  signaling pathway. In conclusion, miR-450 was a new molecular target for the treatment of gastric cancer.

In recent years, researches pay great attention to gastric cancer, trying to find an effective new method to treat gastric cancer. Feng *et al.* reported that miR-518 suppresses the progression of gastric cancer by promoting apoptosis via targeting MDM2 and may be a promising molecular target for treating gastric cancer for the time to come [15]. Liu *et al.* demonstrated that miR-204 modulates the EMT to inhibit the gastric cancer cell migration and invasion via regulating *snai1 in vitro* and *in vivo* [16]. Wang *et al.* proves that miR-129-5p inhibited gastric cancer cell proliferation and EMT by HMGB1, acting as a potential target for curing gastric cancer [17]. Wang *et al.* testifies that overexpression of miR-128b inhibits cell proliferation, migration and invasion, and promoted apoptosis in gastric cancer cells via down-regulation of A2bR [18]. Wu *et al.* reports that up-regulation of miR-449c suppressed gastric cancer cell growth and promoted apoptosis, while down-regulation of miR-449c promoted gastric cancer cell growth and inhibited apoptosis [19]. Wang *et al.* demonstrates that over-expression of miR-217 inhibits gastric cancer cell proliferation, invasion and promotes apoptosis via regulating GPC5, which acts as a potential therapeutic target [20]. Besides, other miRNAs, for instance, miR-376c-3p [21], miR-133b [22], miR-99b-5p and miR-203a-3p [23], miR-491-5p [24], miR-143 [25] are intensively involved in the regulation of the development and progression of gastric cancer. Thus miRNAs is closely associated with the occurrence and development of gastric cancer. Here are some researches indicated that Comparing to the above studies, which suggested that miR-450 was even more specific in gastric cancer.

MiR-21, miR-450 and miR-149 participating in the regulation of glaucoma have been reported [14]. Moreover, miR-21 and miR-149 are associated with the development of gastric cancer [12, 13]. More importantly, given the glaucoma exhibited similar cancer type with gastric cancer, hence, we explored the roles of miR-450 in gastric cancer and miR-450 was confirmed to be a tumor suppressor in gastric cancer. As miR-21 and miR-149 play a role in other various cancers, for instance, colorectal cancer [26], ovarian cancer [27], breast cancer [28], bladder cancer [29], hepatocellular cancer [30], it is worthy to investigate whether miR-450 also plays a role in these cancers. In the following studies, we will further study the role of miR-450 in different cancers, so as to illustrate the role of miR-450 in various cancers, which exhibit the advantages of miR-450 as a molecular therapeutic target.

Researches have reported the AKT/GSK-3 $\beta$  signaling pathway is closely associated to the development and progression of gastric cancer. Hua *et al.* reported that knockdown of ANXA11 suppressed gastric cancer cell proliferation, invasion and migration through the AKT/GSK-3 $\beta$  pathway, which was acted as a prognostic factor and promising molecular target for gastric cancer treatment [31]. Pan *et al.* demonstrated that CD36 regulated palpitae acid-induced metastasis in gastric cancer by AKT/GSK-3 $\beta$ / $\beta$ -catenin pathway, acting as a potential molecular target for clinical intervention [32]. In our study, we found that miR-450 blocked the AKT/GSK-3 $\beta$  signaling pathway to regulate the progression of gastric cancer and miR-450 levels were related with strength of the AKT/GSK-3 $\beta$  signal pathway.

In conclusion, miR-450 targets CREB1 and inactivates the AKT/GSK-3 $\beta$  signaling pathway to inhibit the development and progression of gastric cancer. miR-450 may act as a tumor-suppressor factor of gastric cancer, promising to be a new molecular target for the treatment of gastric cancer.

## Conclusion

miR-450 increased the expression levels of apoptosis-associated proteins, including Caspase-3, Caspase-9 and BAX, while inhibited the expression level of Bcl-2. miR-450 inhibited the gastric cancer cell proliferation, migration and invasion, while induced apoptosis in gastric cancer in vitro. miR-450 targets and negatively regulates the CREB1. Low expression of CREB1 suppressed gastric cancer cell proliferation, migration and invasion and induced apoptosis. miR-450 represses the gastric cancer progression by targeting CREB1 through inhibiting AKT/GSK-3 $\beta$  signaling pathway. The research may provide a new molecular target for the treatment of gastric cancer.

## Abbreviations

abbreviations	Full name
miRNAs	MicroRNAs
BGC	Gastric Cancer Cell
GES	Gastric Epithelial Cell
DMEM	Dulbecco's Modified Eagle's Medium
RT-qPCR	Real-time Quantitative PCR
CCK-8	Cell Counting Kit-8
OD	Optical Density
HE	Hematoxylin-eosin
IHC	Immunohistochemical
3'-UTR	3'-Untranslated Regions

## Declarations

### Ethics approval and consent to participate

The experimental content meets ethical requirements and obtains permission.

### Consent for publication

The authors declare that they have no conflict of interests.

## Availability of data and material

The source of all data and information is true and reliable

## Competing interests

The authors declare that they have no conflict of interests.

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## Conflicts of interest

There are no conflicts of interest.

## Authors' contributions

All authors have contributed enough to the article

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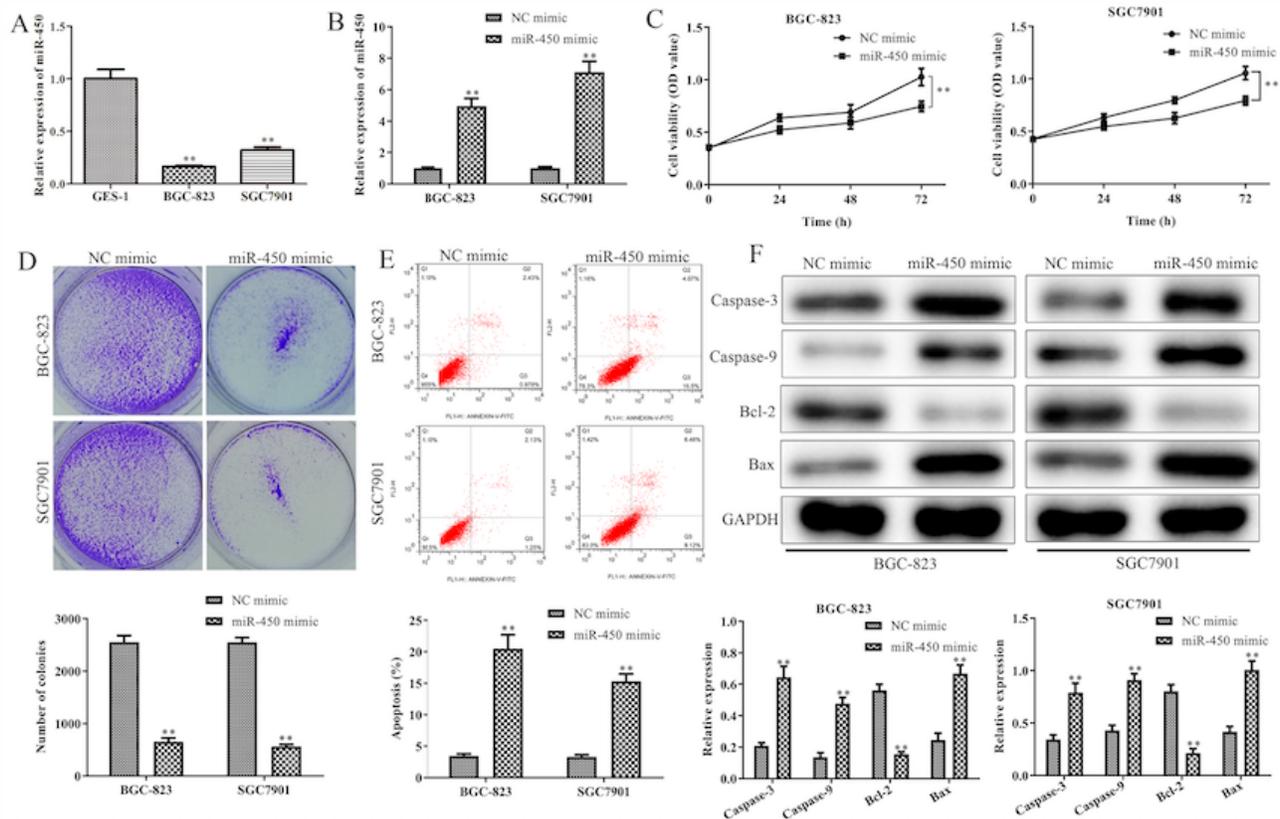
## References

1. Ferlay, J., Soerjomataram, I., Dikshit, R., Eser, S., Mathers, C., & Rebelo, M., et al. (2015). Cancer incidence and mortality worldwide: sources, methods and major patterns in globocan 2012. *Int J Cancer*, 136(5), 359-386.
2. Ferlay, J. (2010). Estimates of worldwide burden of cancer in 2008 :globocan 2008. *Int J Cancer*, 127, 2893-2917.
3. Song, Z., Wu, Y., Yang, J., Yang, D., & Fang, X. (2017). Progress in the treatment of advanced gastric cancer. *Tumor Biol*, 39(7), 101042831771462.
4. Hu, X., Zhang, M., Miao, J., Wang, X., & Huang, C. (2017). MiRNA-4317 suppresses human gastric cancer cell proliferation by targeting ZNF322. *Cell Biol Int*, 42(8), 923-930.
5. Ning, T., Peng, Z., Li, S., Qu, Y., Zhang, H., & Duan, J., et al. (2017). MiR-455 inhibits cell proliferation and migration via negative regulation of EGFR in human gastric cancer. *Oncol Rep*, 38(1), 175-182.
6. Jin, Y., Tao, L. P., Yao, S. C., Huang, Q. K., & Jin, S. Q. (2017). MicroRNA-582-5p suppressed gastric cancer cell proliferation via targeting AKT3. *Eur Revi Med Pharmacol Sci*, 21(22), 5112-5120.
7. Xu, A., Fu, L., Wu, H., Yao, X., & Meng, R. (2017). MicroRNA-744 inhibits tumor cell proliferation and invasion of gastric cancer via targeting brain-derived neurotrophic factor. *Mol Med Rep*, 16(4), 5055-5061.

8. Fang, Z., Yin, S., Sun, R., Zhang, S., Fu, M., & Wu, Y., et al. (2017). MiR-140-5p suppresses the proliferation, migration and invasion of gastric cancer by regulating YES1. *Mol Cancer*, 16(1), 139.
9. Lin, F., Li, Y., Yan, S., Liu, S., Qian, W., & Shen, D., et al. (2015). MicroRNA-181a inhibits tumor proliferation, invasiveness, and metastasis and is downregulated in gastric cancer. *Oncol Res*, 22(2), 75-84.
10. Tang, L., Chen, F., Pang, E. J., Zhang, Z. Q., Jin, B. W., Dong, W. F. (2015). MicroRNA-182 inhibits proliferation through targeting oncogenic ANUBL1 in gastric cancer. *Oncol Rep*, 33(4), 1707-1716.
11. Zhang, X. Y., Mu, J. H., Liu, L. Y., & Zhang, H. Z. (2017). Upregulation of miR-802 suppresses gastric cancer oncogenicity via targeting rab23 expression. *Eur Rev Med Pharmacol Sci*, 21(18), 4071-4078.
12. Wang, P., Guan, Q., Zhou, D., Yu, Z., Song, Y., & Qiu, W. (2018). MiR-21 inhibitors modulate biological functions of gastric cancer cells via pten/GSK-3 $\beta$  /mtor pathway. *DNA Cell Biology*, 37(1), 38-45.
13. Li, X., Liang, J., Liu, Y. X., Wang, Y., Yang, X. H., & Luan, B. H., et al. (2016). MiR-149 reverses cisplatin resistance of gastric cancer SGC7901/DDP cells by targeting FoxM1. *Pharmazie*, 71(11), 640-643.
14. Izzotti, A., Ceccaroli, C., Longobardi, M. G, Micale, R. T., Pulliero, A., La Maestra, S., Saccà, SC. (2015). Molecular Damage in Glaucoma: from Anterior to Posterior Eye Segment. *The MicroRNA Role. Microna*, 4(1), 3-17.
15. Feng, C., Xian, Q., & Liu, S. (2018). MicroRNA-518 inhibits gastric cancer cell growth by inducing apoptosis via targeting MDM2. *Biomed Pharmacother*, 97, 1595-1602.
16. Liu, Z., Long, J., Du, R., Ge, C., & Xu, Y. (2016). MiR-204 regulates the emt by targeting snai1 to suppress the invasion and migration of gastric cancer. *Tumor Biol*, 37(6), 8327-8335.
17. Wang, S., Chen, Y., Yu, X., Lu, Y., Wang, H., Wu, F., Teng, L. (2019). miR-129-5p attenuates cell proliferation and epithelial mesenchymal transition via HMGB1 in gastric cancer. *Pathol Res Pract*. 215(4), 676-682.
18. Wang, P., Guo, X., Zong, W., Song, B., Liu, G., He, S. (2015). MicroRNA-128b suppresses tumor growth and promotes apoptosis by targeting A2bR in gastric cancer. *Biochem Biophys Res Commun*. 467(4), 798-804.
19. Wu, Z., Wang, H., Fang, S., & Xu, C. (2015). MiR-449c inhibits gastric carcinoma growth. *Life Sci*, 137, 14-19.
20. Wang, H., Dong, X., Gu, X., Qin, R., Jia, H., & Gao, J. (2015). The microRNA-217 functions as a potential tumor suppressor in gastric cancer by targeting GPC5. *PLOS ONE*, 10(6), e0125474.
21. Tu, L., Zhao, E., Zhao, W., Zhang, Z., Tang, D., Zhang, Y., Wang, C., Zhuang, C., Cao, H. (2016). hsa-miR-376c-3p Regulates Gastric Tumor Growth Both *In Vitro* and *In Vivo*. *Biomed Res Int*. 2016, 9604257.
22. Guo, L., Bai, H., Zou, D., Hong, T., Liu, J., & Huang, J., et al. (2014). The role of microRNA-133b and its target gene FSCN1 in gastric cancer. *J Exp Clin Cancer Res*, 33(1), 99.
23. Wang, Z., Zhao, Z., Yang, Y., Luo, M., Zhang, M., & Wang, X., et al. (2018). MiR-99b-5p and miR-203a-3p function as tumor suppressors by targeting igf-1r in gastric cancer. *Sci Rep*, 8(1), 10119

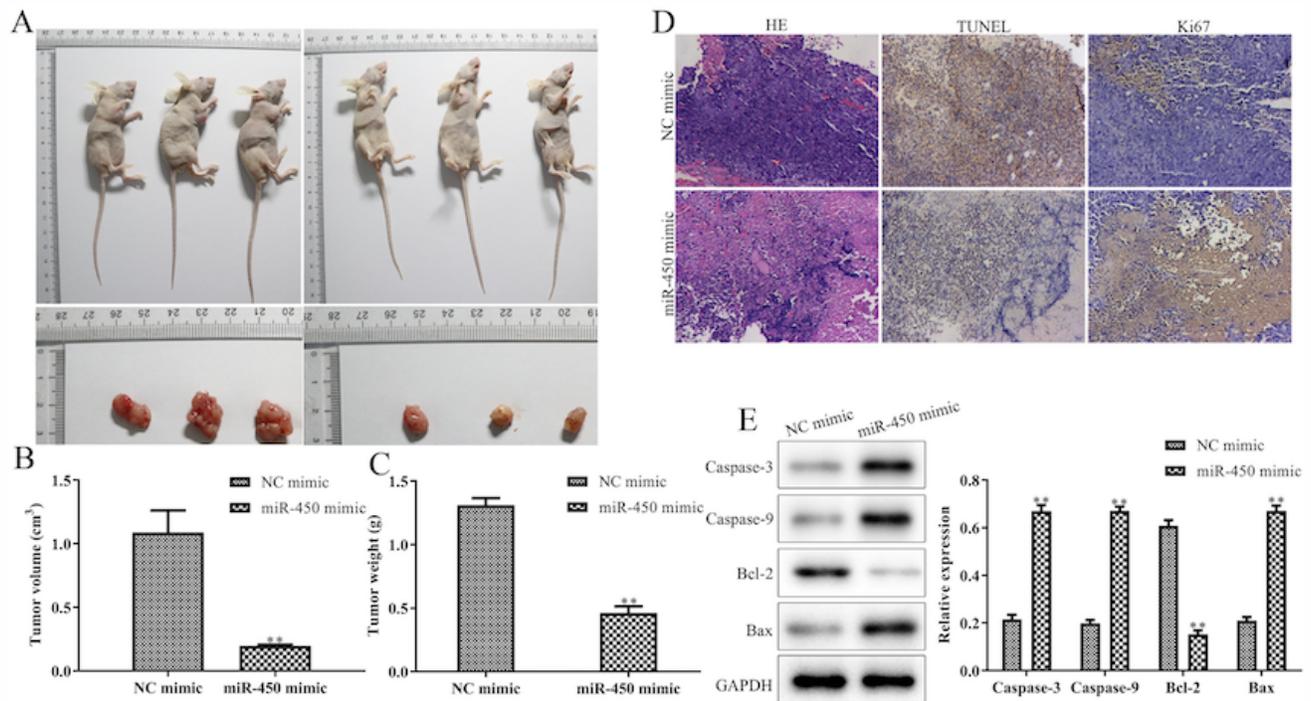
24. Yu, T., Wang, L. N., Li, W., Zuo, Q. F., Li, M. M., & Zou, Q. M., et al. (2018). Downregulation of miR-491-5p promotes gastric cancer metastasis by regulating SNAIL and FGFR4. *Cancer Sci*, 109(5), 1393-1403.
25. Mao, G. P., Liu, R., & Qin, Y. R. (2018). MiR-143 inhibits cell proliferation of gastric cancer cells through targeting GATA6. *Oncol Res*, 6(7), 1023-1029.
26. Wu, Y., Song, Y., Xiong, Y., Wang, X., Xu, K., & Han, B., et al. (2017). MicroRNA-21 (miR-21) promotes cell growth and invasion by repressing tumor suppressor PTEN in colorectal cancer. *Cellular Physio Biochemi*, 43(3), 945-958.
27. Báez-Vega, P. M., Echevarría Vargas, I. M., Valiyeva, F., Encarnación Rosado, J., Marcos-Martínez, M. J., Vivas-Mejía, P. E. (2014). Targeting miR-21-3p inhibits proliferation and invasion of ovarian cancer cells. *Oncotarget*, 7(24), 36321-36337.
28. Petrović (2016). MiR-21 might be involved in breast cancer promotion and invasion rather than in initial events of breast cancer development. *Mol Diagn Ther*, 20(2), 97-110.
29. Yang, D., Du, G., Xu, A., Xi, X., & Li, D. (2017). Expression of miR-149-3p inhibits proliferation, migration, and invasion of bladder cancer by targeting S100A4. *Am J Cancer Rese*, 7(11), 2209-2219.
30. Luo, G., Chao, Y. L., Tang, B., Li, B. S., & Yang, S. M. (2015). MiR-149 represses metastasis of hepatocellular carcinoma by targeting actin-regulatory proteins PPM1F. *Oncotarget*, 6(35), 37808-37823.
31. Hua, K., Li, Y., Zhao, Q., Fan, L., & Gu, J. (2018). Downregulation of Annexin a11 (ANXA11) inhibits cell proliferation, invasion, and migration via the AKT/GSK-3 $\beta$  pathway in gastric cancer. *Med Sci Monit*, 24, 149-160.
32. Pan, J., Fan, Z., Wang, Z., Dai, Q., Xiang, Z., & Yuan, F., et al. (2019). CD36 mediates palmitate acid-induced metastasis of gastric cancer via AKT/GSK-3 $\beta$ / $\beta$ -catenin pathway. *J Exp Clin Cancer Res*, 38(1), 52.

## Figures



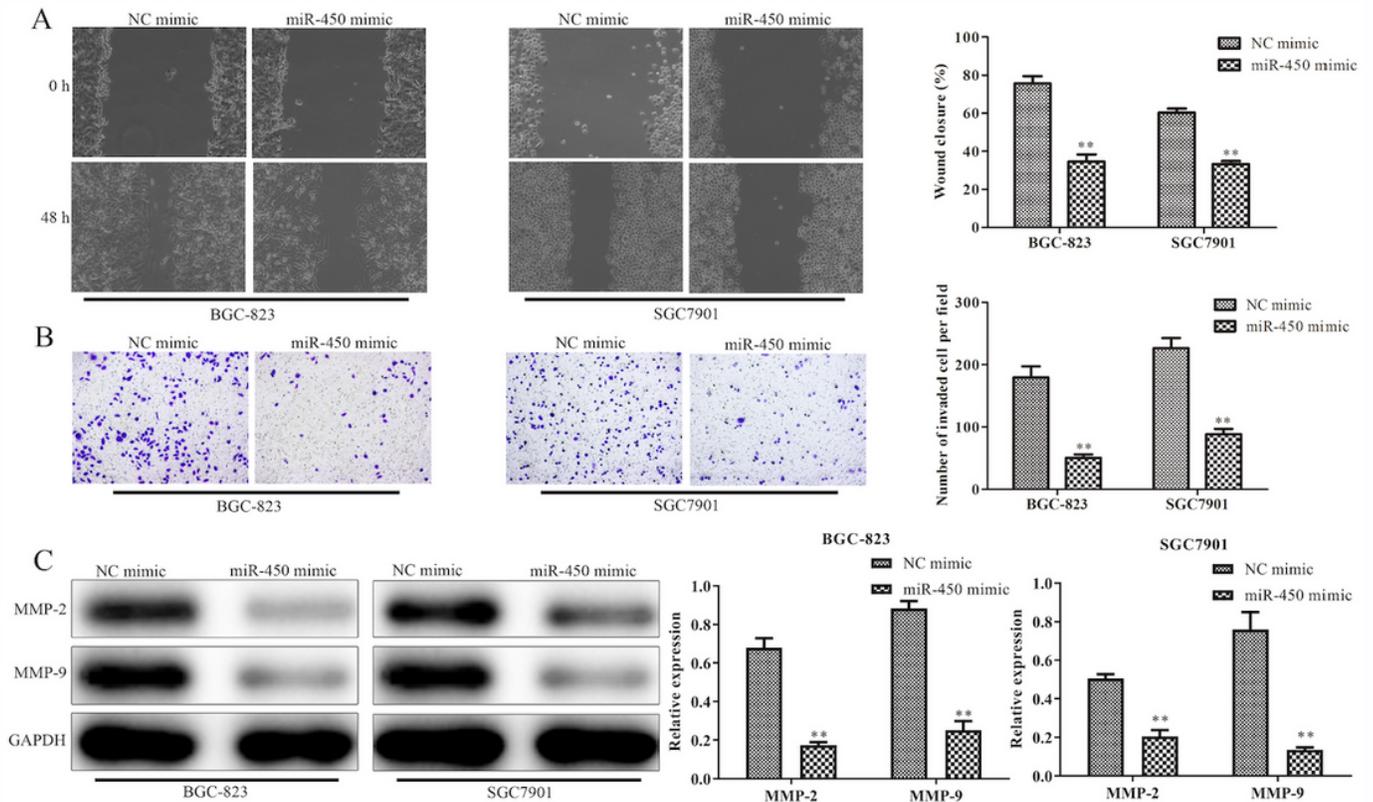
**Figure 1**

Overexpression of miR-450 inhibits on cell proliferation and apoptosis of gastric cancer. (A) The expression levels of miR-450 were measured by RT-qPCR in gastric cancer cell lines (BGC-823 and SGC-7901) and a human gastric epithelial cell line (GES-1). \*\* $P < 0.01$  vs. GES-1 group. (B) The expression levels of miR-450 were assessed by RT-qPCR assay. \*\* $P < 0.01$  vs. miR-NC group. (C-E) CCK-8, colony formation and flow cytometry assays were used to detect the cell proliferation and apoptosis of BGC-823 and SGC-7901 cells. \*\* $P < 0.01$  vs. miR-NC group. (F) Western blot assay was conducted to evaluate the apoptosis-associated protein expression levels in BGC-823 and SGC-7901 cells. \*\* $P < 0.01$  vs. miR-NC group.



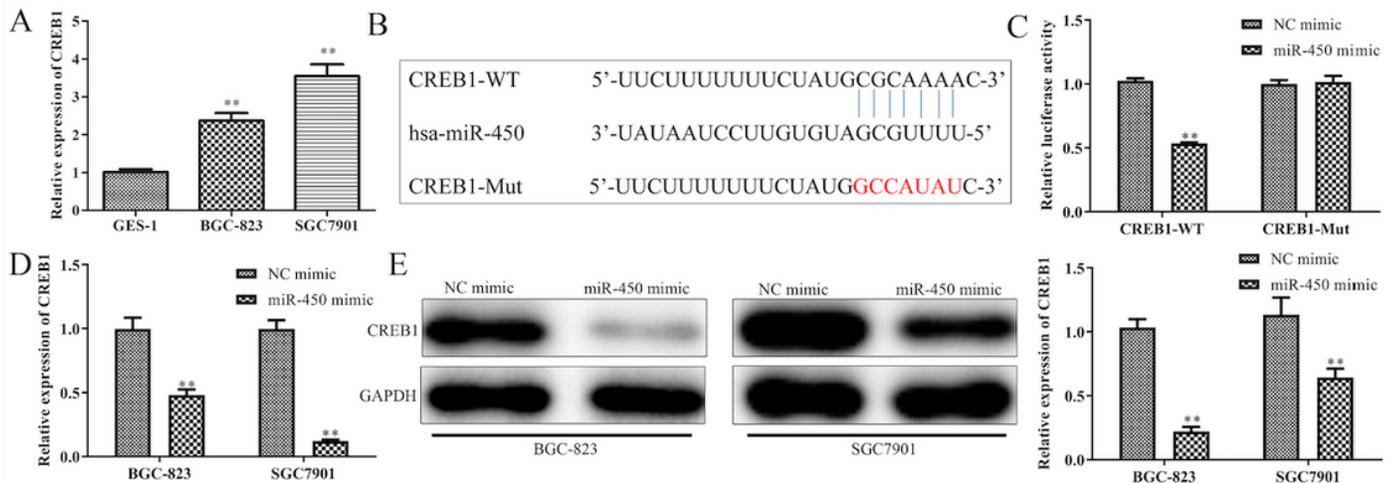
**Figure 2**

Overexpression of miR-450 inhibits tumor growth. (A-C) Tumor phenotype, measured tumor volume and weight were recorded. (D) HE, TUNEL and IHC were performed to detect pathological morphology and the expression levels of Ki67 and apoptosis rate in tumor tissues. (E) Western blot assay was applied to evaluate the apoptosis-associated protein expression levels in tumor tissues. \*\*P < 0.01 vs. miR-NC group.



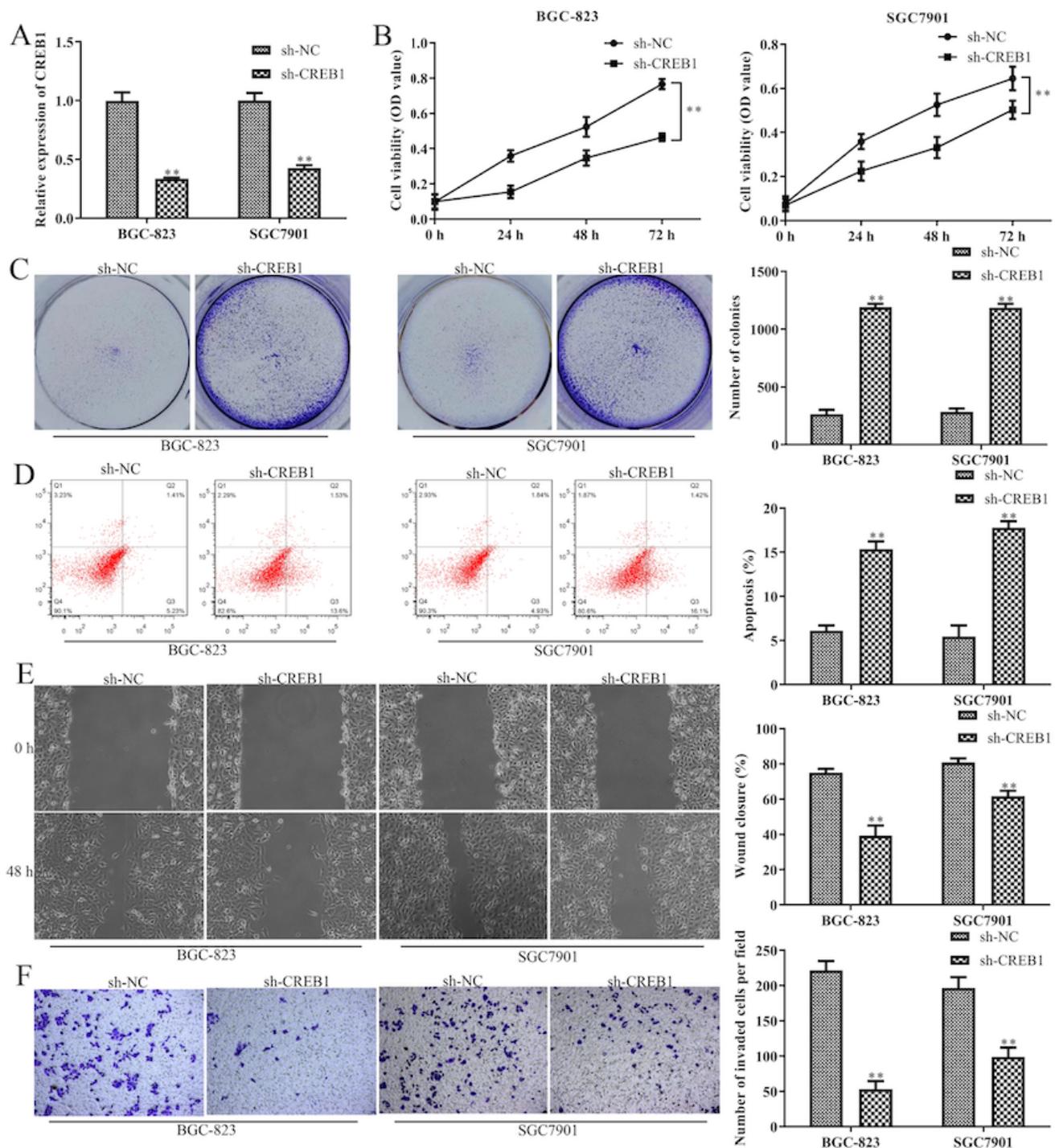
**Figure 3**

Overexpression of miR-450 inhibits gastric cancer cell migration and invasion. (A) Wound healing assay was carried out to confirm cell migration capability in BGC-823 and SGC-7901 gastric cancer cells after transfection of miR-450. (B) Transwell chamber assay was conducted to determine cell invasion capability in BGC-823 and SGC-7901 gastric cancer cells after transfection of miR-450. (C) Western blot assay was performed to study migration- and invasion-associated protein expression levels in BGC-823 and SGC-7901 gastric cancer cells after transfection of miR-450. \*\*P < 0.01 vs. miR-NC group.



## Figure 4

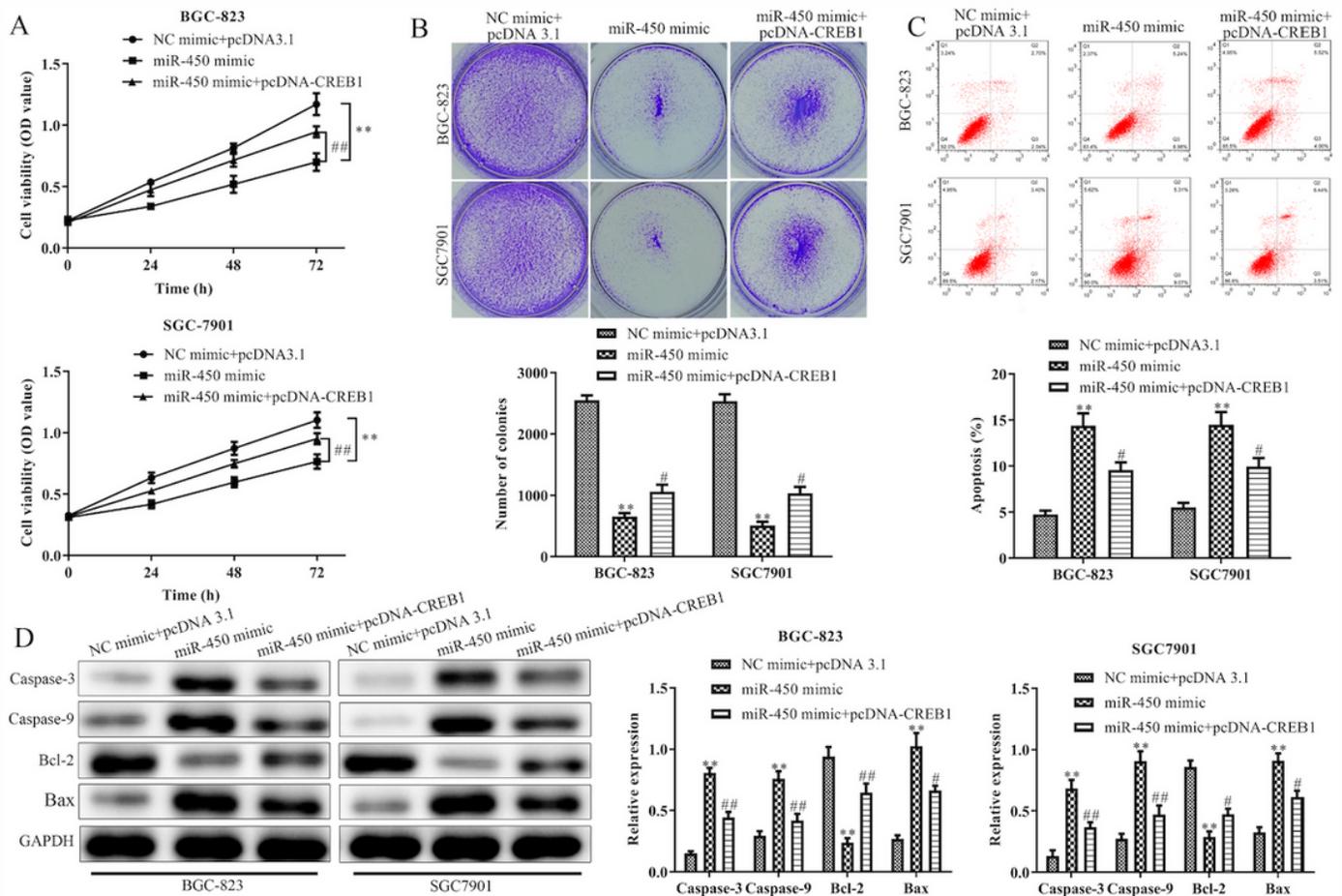
MiR-450 targets and negatively regulates CREB1. (A) The expression levels of CREB1 were determined by RT-qPCR in GES-1, BGC-823 and SGC7801 cells. \* $P < 0.05$  vs. GES-1 group. (B) The binding sites between miR-450 and CREB1 were predicted. (C) Dual luciferase reporter assay was performed in HEK-293T cells to detect the interaction between miR-450 and CREB1. \*\* $P < 0.01$  vs. miR-NC group. (D) RT-qPCR was applied to to examine the CREB1 expression level after transfection of miR-450. \*\* $P < 0.01$  vs. miR-NC group. (E) Western blot assay was carried out to examine the CREB1 expression after transfection of miR-450. \*\* $P < 0.01$  vs. miR-NC group.



**Figure 5**

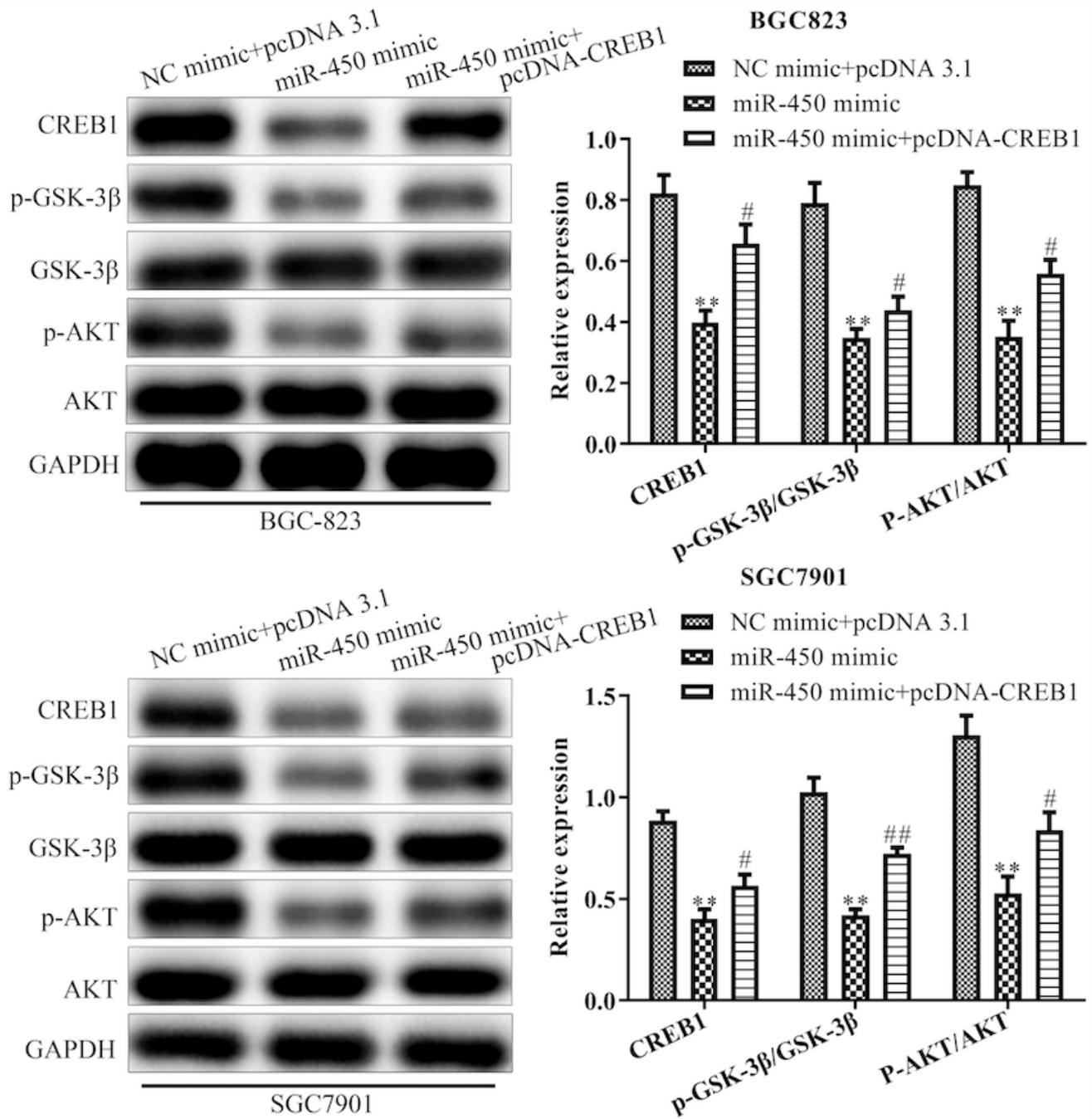
Knockdown of CREB1 affects gastric cancer cell proliferation, apoptosis, migration and invasion. (A) RT-qPCR was performed to evaluate the efficiency of CREB1 knockdown in BGC-823 and SGC-7901 cells. (B-C) CCK-8 and colony formation assays were used to detect the cell proliferation of BGC-823 and SGC-7901 cells after knockdown of CREB1. (D) Flow cytometry was carried out to investigate the cell apoptosis of BGC-823 and SGC-7901 cells after knockdown of CREB1. (E, F) Wound healing and

transwell chamber assays was carried out to confirm the cell migration and invasion of BGC-823 and SGC-7901 cells after knockdown of CREB1.  $**P < 0.01$  vs. sh-NC group.



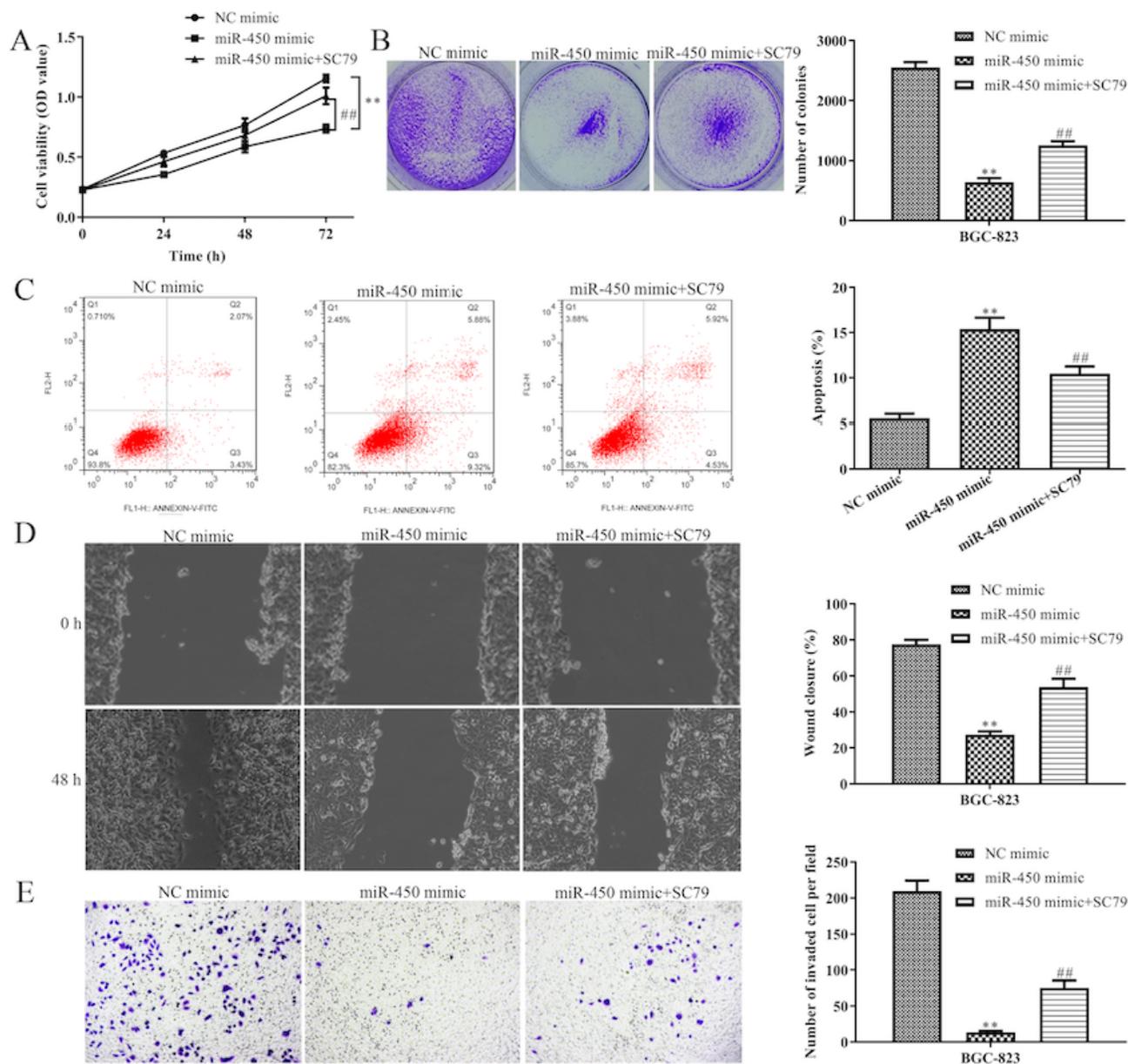
**Figure 6**

CREB1 overexpression partially restores the effects of miR-450 on gastric cancer. (A-B) CCK-8 assay and colony formation assay were performed to detect the cell proliferation of BGC-823 and SGC-7901 cells. (C) Flow cytometry was used to investigate the cell apoptosis of BGC-823 and SGC-7901 cells. (D) Western blot assay was carried out to evaluate the apoptosis-associated protein expression in BGC-823 and SGC-7901 cells.  $**P < 0.01$  vs. NC mimic + pcDNA3.1 group.  $\#P < 0.05$ ,  $\#\#P < 0.05$  vs. miR-450 mimic group.



**Figure 7**

MiR-450 regulates the AKT/GSK-3β signaling pathway. Western blotting was conducted to evaluate the protein expression of CREB1, GSK-3β, AKT, p-GSK-3β, p-AKT. \*\*P < 0.05 vs. NC mimic + pcDNA3.1 group. #P < 0.05, ##P < 0.01 vs. miR-450 mimic group.



**Figure 8**

MiR-450 inhibits the progression of gastric cancer by regulating the AKT/GSK-3 $\beta$  signaling pathway. (A-B) CCK-8 and colony formation assays were performed to detect the cell proliferation of BGC-823 cells. (C) Flow cytometry was used to investigate the cell apoptosis of BGC-823 cells. (D) Wound healing assay was carried out to confirm the cell migration of BGC-823 cells after knockdown of CREB1. (E) Transwell chamber assay was conducted to determine the cell invasion of BGC-823 cells after knockdown of CREB1. \*\* $P < 0.01$  vs. NC mimic group. ## $P < 0.01$  vs. miR-450 mimic group.